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Detection of Three Potato Viruses Isolated from Saudi Arabia

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ABSTRACT

Potato Leaf Roll Virus (PLRV), Potato Virus Y (PVY) and Potato Virus X (PVX) are the most economically important viruses in commercial potato production. Enzyme Linked Immunosorbent Assay (ELISA) and the Reverse Transcription Polymerase Chain Reaction (RT-PCR) were used for the detection of these three potato viruses. Specific primer pairs designed to amplify the coat protein gene of each virus (627 bp for PLRV, 801 bp for PVY and 714 bp for PVX) were successfully applied. A multiplex RT-PCR (mRT-PCR) was developed for the simultaneous detection of the three viruses in potato leaves. The nucleotide sequences of the coat protein genes of the Saudi isolate of PLRV (PLRV SA3), the Saudi isolate of PVY (PVY SA2) and the Saudi isolate of PVX (PVX SA1) were submitted in the GenBank under accession numbers: KC875235, KC875237 and KC875236, respectively. The nucleotide sequences PLRV SA3, PVY SA2 and PVX SA1 were compared to the sequences of the coat protein genes of other PLRV, PVY and PVX isolates. The similarity of the nucleotide sequences suggested that the architecture of the polerovirus (PLRV), potyvirus (PVY) and potexvirus (PVX) are highly conserved. This study describes an assay where three common potato-infecting viruses, Potato leafroll virus, Potato virus Y and Potato virus X, were detected simultaneously from total RNA potato leaves in a multiplex RT-PCR.

Key words: *Solanum tuberosum*, PLRV, PVY, PVX, mRT-PCR, Saudi Arabia

INTRODUCTION

Production of the potato in the developing world has increased faster in recent years than any other major food crop. Potato is considered one of the most important vegetable crops in Saudi Arabia. Potato acreage has increased several times and this expansion was mainly due to government policy of diversifying food production and reducing wheat acreage (Duwais *et al.*, 1999). Like most vegetatively propagated crops, potato suffers from many yield-limiting diseases. A wide range of diseases, including fungal, viral, viroid and nematode diseases infect potato (Salazar, 1977).

Potato leafroll virus (PLRV; genus *Polerovirus*, family Luteoviridae), like all other luteoviruses, has a mono-partite, single stranded RNA genome. PLRV is transmitted by aphids in a circulative non-propagative manner and is mainly restricted to phloem tissues of infected plants (Mayo and Ziegler-Graff, 1996).

Potato virus Y (PVY) is an important pathogen in solanaceous crops. It belongs to the genus *Potyvirus*, of which it is the type species, in the plant virus family Potyviridae (Shukla *et al.*, 1994). PVY has a single positive-sense RNA. It is naturally transmitted by aphids in a non-persistent manner, causing epidemics in potato, tomato, pepper, tobacco and other solanaceous plants (De Bokx and Huttinga, 1981).

Potato virus X (PVX) a type member of the potexvirus family, is a rod-shaped virus containing single stranded RNA capped, polyadenylated and contains five Open Reading Frames (ORFs) (Huisman *et al.*, 1988). PLRV, PVY and PVX are the most prominent viruses in the field with some of their isolates inflicting heavy economic losses in potato cultivations worldwide (Salazar, 1996; Warren *et al.*, 2005; Bawden *et al.*, 1948).

Potato production in central Saudi Arabia suffers from the common potato viruses such as PVY, PVS and PLRV (Al-Shahwan *et al.*, 1998). Standard methods of virus detection including mechanical inoculation to indicator hosts, electron microscopy, ELISA and visual inspection are not adequate for virus detection in potato tubers (Mumford *et al.*, 2000). Increased sensitivity and specificity of detection offered by RT-PCR is an effective method to overcome these problems (Nie and Singh, 2002). Multiplex polymerase chain reaction (m-PCR) accommodates several pairs of primers in one reaction, resulting in reduced material costs and time when compared to several individual PCR reactions (Shalaby *et al.*, 2002). PLRV, PVA, PVX and PVY, were detected simultaneously from total RNA and sap of dormant potato tubers in a quadruplex real-time RT-PCR (Agindotan *et al.*, 2007). This study aimed to use molecular biology tools for identification and characterization of the three potato viruses (PLRV, PVY and PVX) isolated from Saudi Arabia.

MATERIALS AND METHODS

All work was done at Pest and Plant Diseases Unit (PPDU), College of Agricultural and Food Sciences, King Faisal University, Saudi Arabia.

Source of isolates: Potato samples (leaves) suspected to be infected with potato viruses were collected from two locations of The National Agricultural Development Company (NADEC) farms at Harad which located at the east of Saudi Arabia (variety; Hermes) and at Hail which located at the middle north of Saudi Arabia (varieties; Hermes and Lady Olympia). Sixty-one samples were collected from Harad and 66 samples were collected from Hail.

ELISA: ELISA tests for the detection of potato viruses (PLRV, PVY and PVX) in the collected potato samples were carried out following the method described by ELISA kits (Bioreba AG, USA).

Primers for PLRV, PVY and PVX: Pair of primers specific for PLRV coat protein gene (PLRV-CP), PVY coat protein gene (PVY-CP) and PVX coat protein gene (PVX-CP) were used for the CP genes according to Shalaby *et al.* (2002). The forward primer PLRVCPvEcoRI with *EcoRI* restriction enzyme site (underlined) at the 5' end and the complementary primer PLRVCPcNcoI with *NcoI* restriction enzyme site (underlined) at the 5' end were used for the detection of coat protein gene of PLRV (Table 1). The forward primer PVYCPvBamHI with *BamHI* restriction site (underlined) at the 5' end and the complementary primer PVYCPcEcoRI with *EcoRI* restriction enzyme site (underlined) at the 5' end were used for the detection of coat protein gene of PVY (Table 1). The forward primer PVXCPvEcoRI with *EcoRI* restriction enzyme site (underlined) at the 5' end and the complementary primer PVXCPcNcoI with *NcoI* restriction enzyme site (underlined) at the 5' end were used for the detection of coat protein gene of PVX (Table 1).

One step RT-PCR: One step RT-PCR was done using "Platinum Quantitative RT-PCR Thermo Script One Step System" (Invitrogen, USA) on the samples which gave positive reaction in ELISA test. Total RNA extracted from infected potato plants using RNeasy[®] plant Mini Kit (Qiagen, USA) were used as templates for one-tube RT-PCR amplification reactions. RT-PCR mixture prepared by

Table 1: Sequences of the specific primers used to detect the coat protein genes of potato viruses PLRV, PVY and PVX

Viruses	Primer's name	Nucleotide sequences	Size (bp)
PLRV	PLRVCPvEcoRI	5'-AATAGAATTCTAATGAGTACGGTCGTGGTTARAGG-3'	650
	PLRVCPeNcoI	5'-AAAACCATGGCTATYTTGGGGTTYTG CARAGCTAC-3'	
PVY	PVYCPvBamHI	5'-TCAAGGATCCGCAAATGACACAATTGATGCAGG-3'	825
	PVYCPeEcoRI	5'-AGAGAGAATTCATCACATGTTCTTGACTCC-3'	
PVX	PVXCPvEcoRI	5'-GATAGAATTCAGATGACTACACCAGCCAACACC-3'	750
	PVXCPeNcoI	5'-TACGCGTCGGTTCATGGACGTAGTTATGGTGG-3'	

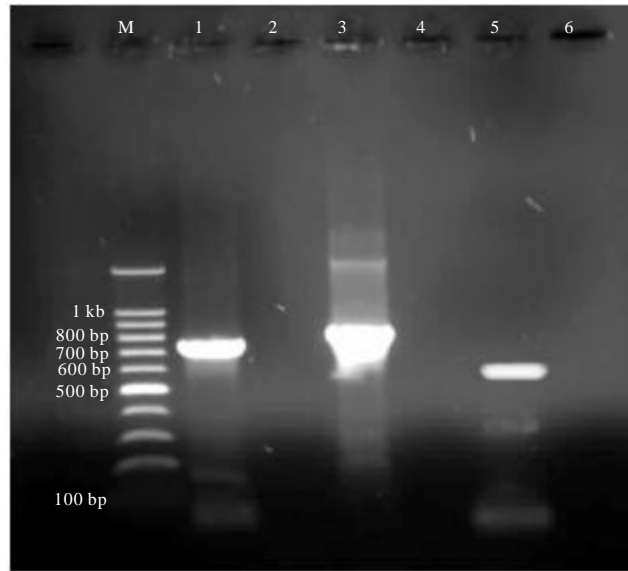


Fig. 1: Agarose gel electrophoresis of RT-PCR products of potato viruses using primers for coat protein genes. M: 100 bp DNA ladder, 1: Sample infected with PVX (750 bp), 3: Sample infected with PVY (825 bp), 5: Sample infected with PLRV (650 bp) while 2, 4 and 6: Empty well

combining 12.5 μ L of 2 X ThermoScript Reaction Mix, 5 μ L of total RNA, 1 μ L of 10 μ M of each primer (forward and reverse primers; Table 1), 0.5 μ L of ThermoScript *Taq* Enzyme Mix and the reaction was completed to 25 μ L with double distilled water. Reverse transcription reaction started with incubation at 50°C for 30 min, followed by denaturation at 95°C for 5 min. PCR amplification was performed by 35 cycles in a thermal cycler starting with denaturation at 95°C for 1 min, primer annealing at 55°C for 1 min and extension at 72°C for 1 min with final extension at 72°C for 10 min. Five microliters aliquots of RT-PCR products were analyzed on 1% agarose gels in 0.5X TBE buffer (Fig. 1).

Multiplex one step RT-PCR: Multiplex one step RT-PCR was done using “Platinum Quantitative RT-PCR Thermo Script One Step System” (Invitrogen, USA) as described before using 1 μ L of 10 μ M of each primer of the three potato viruses (forward and reverse primers; Table 1) with the same PCR program described above. Five μ L aliquots of RT-PCR products were analyzed on 1% agarose gels in 0.5X TBE buffer (Fig. 2).

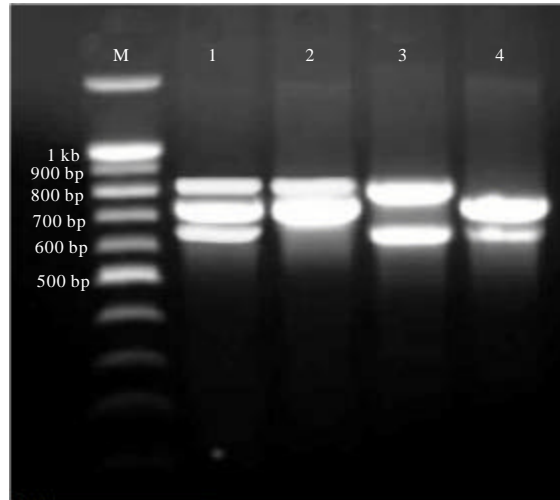


Fig. 2: Agarose gel electrophoresis of CP of potato viruses using multiplex RT-PCR. M: 100 bp DNA ladder, 1: PVY (825 bp), PVX (750 bp) and PLRV (650 bp), 2: PVY and PVX, 3: PVY and PLRV, 4: PVX and PLRV

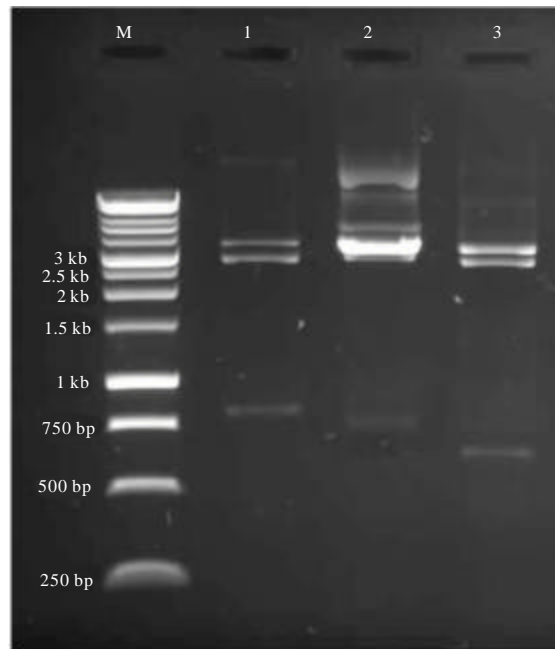


Fig. 3: Enzyme digestion of the recombinant plasmids from the cloning of the RT-PCR amplified products. M: 1 Kb DNA ladder; 1, 2 and 3: DNA minipreps of PVY-CP, PVX-CP and PLRV-CP, respectively in pGEM[®]-T-easy vector digested with *EcoRI*

Cloning and sequencing of the RT-PCR products: The RT-PCR product was ligated into pGEM[®]-T Easy vector (Promega, USA) and the recombinant plasmids were introduced into *E. coli* strain DH5a as described by Sambrook *et al.* (1989). Recombinant plasmids were extracted from selected white colonies using Wizard Plus SV Minipreps DNA Purification System (Promega, USA) digested with *EcoRI* and fractionated on agarose gels using 1% agarose gel in 0.5X TBE buffer (Fig. 3). The nucleotide sequence of clones having expected inserts were selected for dideoxy

sequencing with ABI 377XL automated DNA sequencing instrument, using 36 cm well to read plates and a 5% Long Ranger (FMC) acrylamide gel. Data were analyzed using ABI™ version 3.0 of sequencing analysis. All sequencing procedures were completed in Macrogen Company (South Korea). The nucleotide sequences of the coat protein genes of potato viruses (PLRV, PVY and PVX) were compared and analyzed using DNAMAN Sequence Analysis Software (Lynnon BioSoft, Quebec, Canada) with those of other isolates available in GenBank.

RESULTS AND DISCUSSION

ELISA test: The ELISA tests were done on 127 samples (leaves) collected from Harad and Hail using specific antibodies for PLRV, PVY and PVX (Bioreba, USA) and the results of the experiments represented in Table 2.

From Table 2 we can conclude that, 35 samples were positive with PLRV antibody against this virus with 27.56% of infection. Four samples were positive with PVY antibody against the virus with 3.15% of infection. Two samples were positive with PVX antibody against this virus with 1.57% of infection.

The most common method for the detection of plant viruses is ELISA because it is rapid and inexpensive. However, the serological methods have the drawbacks of limited availability of antisera (Peiman and Xie, 2006). Many investigators have used ELISA test for serological diagnosis of PLRV, PVY and PVX, Varveri (2006), Li *et al.* (2006) and Boukhris-Bouhachem *et al.* (2007).

Amplification of the potato viruses CP genes: RT-PCR amplification of viral RNAs were carried out on the total RNAs isolated from infected plants using specific primers designed to amplify the coat protein genes. Electrophoresis analysis of RT-PCR products showed single amplified fragments of 750, 825 and 650 bp for PVX-CP, PVY-CP and PLRV-CP, respectively. No fragments were amplified from the RNAs extracted from symptomless or healthy plants (Fig. 1).

Multiplex one step RT-PCR: Multiplex one step RT-PCR amplification of viral RNAs were carried out on the total RNAs isolated from infected plants using specific primers designed to amplify the coat protein genes. Electrophoresis analysis of RT-PCR products showed three amplified fragments (Fig. 2) at lane 1 of 825, 750 and 650 bp for PVY-CP, PVX-CP and PLRV-CP, respectively two amplified fragments at lane 2 of 825 and 750 bp for PVY-CP and PVX-CP, respectively two amplified fragments at lane 3 of 825 and 650 bp for PVY-CP and PLRV-CP, respectively two amplified fragments at lane 4 of 750 and 650 bp for PVX-CP and PLRV-CP, respectively.

The mRT-PCR has a significant advantage in that it permits simultaneous amplification of several viruses in a single reaction (Park *et al.*, 2005; James *et al.*, 2006). Our research demonstrates that the coat protein genes of PLRV, PVY and PVX are reliable regions of the virus genome for the detection of these viruses using mRT-PCR as described by Peiman and Xie (2006).

Table 2: Number of positive samples, negative samples and percentage of infection of all collected samples of potato from Harad and Hail

Potato viruses	No. of samples	No. of samples		Infection(%)
		Positive (+)	Negative (-)	
PLRV	127	35	92	27.56
PVY	127	4	123	3.15
PVX	127	2	125	1.57

Cloning of the potato viruses coat protein genes: The pGEM[®]-T Easy Vector System is convenient system for the cloning of PCR products. The vector is prepared by cutting the pGEM[®]-T Easy Vector with *EcoRV* and adding a 3' terminal thymidine to both ends. These single 3'-T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermo-stable polymerases. These polymerases often add a single deoxyadenosine, in a template-independent fashion, to the 3'-ends of the amplified fragments.

Isolation of recombinant plasmids and enzyme digestion: Several white colonies resistant to ampicillin were selected to test for recombinant plasmids containing the PLRV-CP, PVY-CP and PVX-CP genes. Restriction enzyme digestion with *EcoRI* released the cloned gene due to the presence of *EcoRI* site at both 5' and 3' end in the pGEM[®]-T Easy vector (before and after the position of inserted PCR) (Fig. 3).

Nucleotide sequencing analysis: Nucleotide sequencing of the RT-PCR amplified fragment in the recombinant plasmid for the PLRV-CP, PVY-CP and PVX-CP were completed to determine if this RT-PCR fragments were from the polerovirus, potyvirus and potexvirus groups or not and to compare the sequences from these isolates with those of other sequences of potato-infecting polerovirus, potyvirus and potexvirus groups available in GenBank. The nucleotide sequence of the coat protein gene of the Saudi isolate of PLRV (PLRV SA3) was submitted in the GenBank under Accession No. KC875235. The predicted PLRV-CP gene is 627 nucleotides in size, starting from ATG start codon (methionine), as obtained by comparison with other PLRV sequences and ending with a TAG stop codon from which the 3' NCR (non-coding region) ends. The CP gene codes for a 209 amino acid protein giving a molecular weight of 23 KDa. The nucleotide sequence of the coat protein gene of the Saudi isolate of PVY (PVY SA2) was submitted in the GenBank under Accession No. KC875237. The predicted PVY-CP gene is 801 nucleotides in size, starting from ATG start codon (methionine), as obtained by comparison with other PLRV sequences and ending with a TGA stop codon from which the 3' NCR (non-coding region) ends. The CP gene codes for a 267 amino acid protein giving a molecular weight of 30 KDa.

The nucleotide sequence of the coat protein gene of the Saudi isolate of PVX (PVX SA1) was submitted in the GenBank under Accession No. KC875236. The predicted PVX-CP gene is 714 nucleotides in size, starting from ATG start codon (methionine), as obtained by comparison with other PVX sequences and ending with a TAA stop codon from which the 3' NCR (non-coding region) ends. The CP gene codes for a 238 amino acid protein giving a molecular weight of 25 KDa. Sequence comparisons showed the percentage of similarity ranged from 95-99% of the eight reported isolates of PLRV with the Saudi isolate. The results indicated that the highest sequence similarity was found between PLRVSA3 isolate and PLRV isolates from France, Pakistan, Czech Republic, India, Iran and China at 99%, while the lowest was found with PLRV isolate from Egypt at 95% (Fig. 4).

The similarity of the nucleotide sequences suggested that the architecture of the polerovirus is highly conserved. Multiple sequence alignment of the nucleotide sequence of the coat protein gene of PLRV [Saudi isolate (KC875235)] with the corresponding sequence of eight different PLRV isolates available in GenBank [France (AF453388); Pakistan (AY307123); Czech Republic (EU717545); India (AF539791); Iran (DQ269981); China (DQ309064); Jordan (EU073861) and Egypt (GQ376029)] were analyzed using DNAMAN software (Table 3).

Sequence comparisons showed the percentage of similarity ranged from 91-99% of the six reported isolates of PVY with the Saudi isolate. The results indicated that the highest sequence

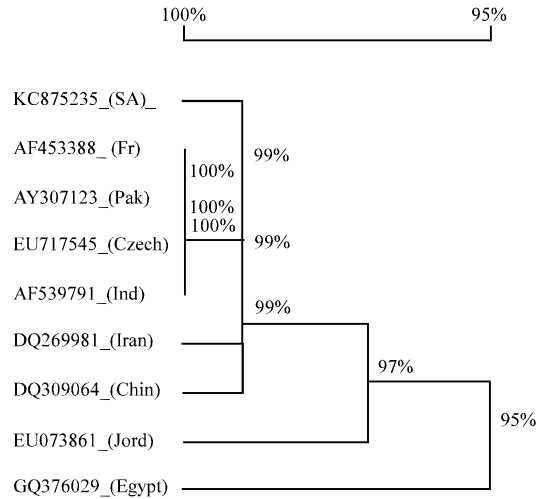


Fig. 4: Phylogenetic tree showing relationships among reported isolates of PLRV and the Saudi isolate (PLRV SA3) based on the nucleotide sequences of their CP genes. Horizontal distances indicate degree of relatedness

Table 3: GenBank accession numbers of PLRV-CP gene sequences used to construct the phylogenetic tree

Accession No.	Country	Host plant	References
KC875235	Saudi Arabia	Potato	This study
AF45338	France	Potato	Guyader and Ducray (2002)
AY307123	Pakistan	Potato	Asad <i>et al.</i> (2003)
EU717545	Czech Rep.	Potato	Plchova <i>et al.</i> (2009)
AF539791	India	Potato	Chakrabarti <i>et al.</i> (2002)
DQ269981	Iran	Potato	Pooramini <i>et al.</i> (2006)
DQ309064	China	Potato	Ding <i>et al.</i> (2005)
EU073861	Jordan	Potato	Odeh and Anfoka (2007)
GQ376029	Egypt	Potato	El-Attar <i>et al.</i> (2010)

Table 4: GenBank accession numbers of PVY-CP gene sequences used to construct the phylogenetic tree

Accession No.	Country	Host plant	References
KC875237	Saudi Arabia	Potato	This study
JQ954387	France	Potato	Cuevas <i>et al.</i> (2012)
GU980964	Egypt	Potato	Soliman <i>et al.</i> (2006)
JN711118	China	Tobacco	Dai <i>et al.</i> (2012)
AB719459	Japan	Potato	Yamamoto and Senda (2012)
JQ518267	Pakistan	Potato	Abbas <i>et al.</i> (2012)
AF325928	UK	Potato	Boonham <i>et al.</i> (2001)

similarity was found between PVYSA2 isolate and PVY isolates from Egypt and France at 99%, while the lowest was found with PVY isolates from Japan, Pakistan and UK at 91% (Fig. 5).

The similarity of the nucleotide sequences suggested that the architecture of the potyvirus is highly conserved. Multiple sequence alignment of the nucleotide sequence of the coat protein gene of PVY [Saudi isolate (KC875237)] with the corresponding sequence of six different PVY isolates available in GenBank [France (JQ954387); Egypt (GU980964); China (JN711118); Japan (AB719459); Pakistan (JQ518267) and UK (AF325928)] were analyzed using DNAMAN software (Table 4).

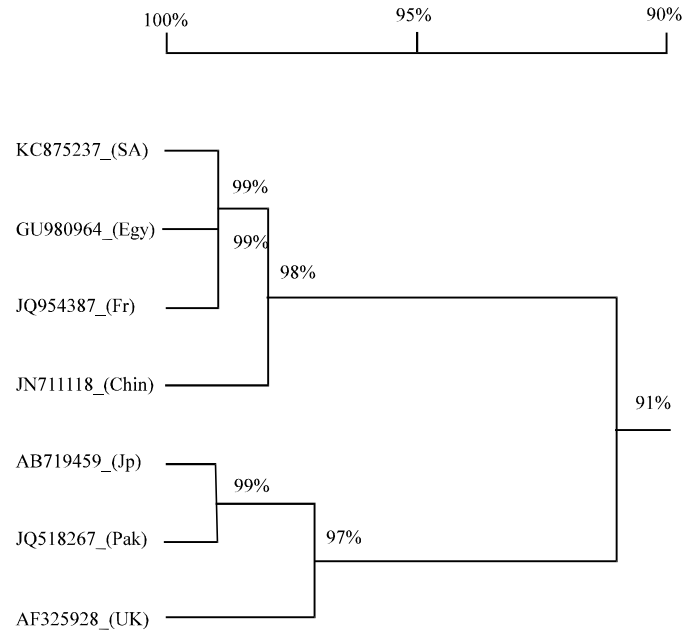


Fig. 5: Phylogenetic tree showing relationships among reported isolates of PVY and the Saudi isolate (PVY SA2) based on the nucleotide sequences of their CP genes. Horizontal distances indicate degree of relatedness

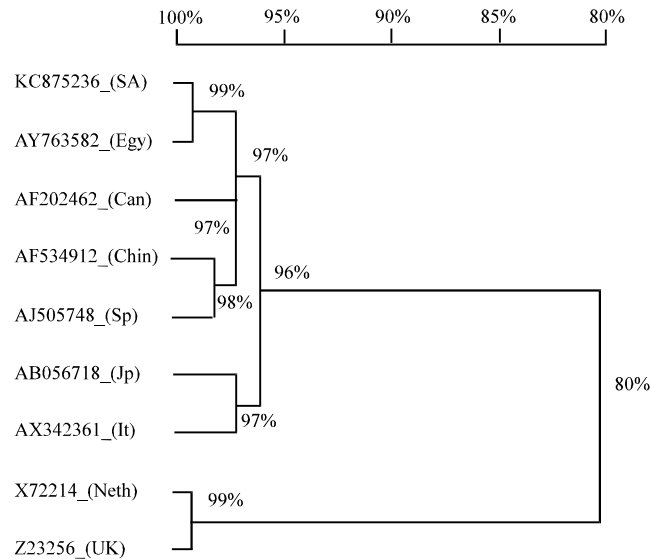


Fig. 6: Phylogenetic tree showing relationships among reported isolates of PVX and the Saudi isolate (PVX SA1) based on the nucleotide sequences of their CP genes. Horizontal distances indicate degree of relatedness

Sequence comparisons showed the percentage of similarity ranged from 80-99% of the eight reported isolates of PVX with the Saudi isolate. The results indicated that the highest sequence similarity was found between PVX SA1 isolate and PVX isolate from Egypt at 99%, while the lowest was found with PVX isolates from Netherland and UK at 80% (Fig. 6).

Table 5: GenBank accession numbers of PVX-CP gene sequences used to construct the phylogenetic tree

Accession No.	Country	Host plant	Reference
KC875237	Saudi Arabia	Potato	This study
AY763582	Egypt	Potato	Soliman <i>et al.</i> (2006)
AF202462	Canada	Potato	Labrosse <i>et al.</i> (1999)
AF534912	China	Potato	Chen and Du (2002)
AJ505748	Spain	Tomato	Martin-Bretones <i>et al.</i> (2002)
AB056718	Japan	Potato	Kagiwada <i>et al.</i> (2002)
AX342361	Italy	Potato	Benvenuto <i>et al.</i> (2002)
X72214	Netherland	Potato	Querci <i>et al.</i> (1993)
Z23256	UK	Potato	Goulden <i>et al.</i> (1993)

The similarity of the nucleotide sequences suggested that the architecture of the potexvirus is highly conserved. Multiple sequence alignment of the nucleotide sequence of the coat protein gene of PVX [Saudi isolate (KC875236)] with the corresponding sequence of eight different PVX isolates available in GenBank [Egypt (AY763582); Canada (AF202462); China (AF594312); Spain (AJ505748); Japan (AB056718); Italy (AX342361); Netherland (X72214) and UK (Z23256)] were analyzed using DNAMAN software (Table 5).

CONCLUSION

The production of virus-free seed potatoes is one of the most important steps in potato production and diagnostic assays to detect plant viruses are needed to assist the generation of high health planting material. In this study we have shown the increased sensitivity of RT-PCR over ELISA, for the detection of PLRV, PVY and PVX in potato leaves. We have also demonstrated the increased efficiency of mRT-PCR, which can detect all three viruses individually in one reaction.

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